Approaches to Gene Transfer in Keratinocytes

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The introduction and expression of exogenous genetic material in cultured cells has provided a powerful tool for studying gene function and regulation. Immortalized cell lines have been useful for establishing gene transfer methodologies that are generally inefficient. For investigators of epidermal and mucosal biology, wishing to make use of the tissue architecture produced by primary keratinocytes *in vitro*, the limited life span of these cells presents a host of unique problems. Primary cells require the use of gene transfer methods that are highly efficient and will not significantly alter the cell's normal differentiation pathway. The purpose of this review is to evaluate gene transfer technology as it applies to keratinocytes. *Key words: epithelia/transfection/transduction. J Invest Dermatol 103:70S–75S, 1994*

A variety of gene transfer procedures have been developed in recent years, some of which have already been used with keratinocytes. In general, genes are delivered into target cells using chemical, physical, or biologic methods **(Table I)**. Chemical approaches rely on the ability of DNA-containing particles to adhere to the surface of cells and to penetrate the cell membrane by endocytosis. Physical methods employ different means to create disruptions in the cell membrane to allow the entrance of genetic material into the cytoplasm. Alternatively, physical methods that disrupt membranes, such as microinjection, allow insertion of DNA directly into the cell nucleus. Biologic gene transfer techniques utilize viral vectors that adhere to specific cellular surface receptors and subsequently become internalized. These approaches have been used to genetically alter keratinocytes *in vitro* and *in vivo*.

Keratinocytes have several characteristics that make them well suited as target cells for gene transfer. *In vitro* culture conditions for the growth and proliferation of keratinocytes have been well established [1] and have produced an intact tissue suitable for grafting [2]. The long-term success of cultured epithelial autografts in treating burn patients suggests that epidermal stem cells are present in culture [3]. A variety of keratinocyte model systems are available for testing the expression of genes during tissue maturation [4]. Keratinocytes in these culture systems are capable of terminal differentiation and of mimicking many of the biochemical and genetic properties of intact epidermis. Finally, if the transferred gene encodes a secreted protein, cultured keratinocytes have been shown to secrete both *in vitro* and after grafting [5].

GENE TRANSFER TECHNIQUES

Chemical Techniques

DNA-Mediated Transfection: A simple procedure that has been used to introduce a gene carried by a plasmid vector into cultured keratinocytes is the incubation of cells with divalent cations such as calcium [6] or strontium. The use of strontium was developed for mouse keratinocytes, which undergo terminal differentiation in the presence of high calcium but are insensitive to strontium [7]. Using DNA-mediated transfection, as many as 50% of the cells can take up DNA but generally only 1–10% will express the transfected gene [8]. Although the mechanism is not clearly understood, it is believed that transfected DNA enters the cytoplasm where it is often destroyed by lysosomal enzymes before it can reach the nucleus. Agents such as chloroquine are helpful in stabilizing lysosomes and are often added for this purpose [9].

In the cases where the cloned DNA does enter the nucleus it usually persists and is expressed for several days but is eventually lost, this phenomenon is referred to as transient expression. In a small subpopulation of the transfected cells (between 1×10^{-3} and 1×10^{-6}) the DNA becomes incorporated into the genome permanently altering the cell resulting in stable expression. The integration event that causes stable expression of the newly introduced DNA is often accompanied by variability in copy number, deletions, and rearrangements. Co-transfection with a dominant drug resistance gene, such as the gene encoding for neomycin B resistance (neo^r) [10] or hygromycin resistance [11], allows recovery of the rare, stably transformed cells. This system has been exploited in keratinocyte lines [12,13] as well as in primary keratinocytes [14,15].

Calcium-mediated DNA transfer has been compared to DEAE dextran- and polybrene-mediated transfection as far as effectiveness in introducing DNA into keratinocytes [16]. In the study of Jiang *et al* complexing the DNA with polybrene or lipids resulted in higher levels of transient gene expression than using calcium mediated gene transfer. However, irrespective of the gene transfer method, expression usually diminishes within several days. For this reason, these methods have seldom been successful for stable expression of exogenous DNA introduced into keratinocytes [17].

Lipofection: The use of artificial phospholipid vesicles or liposomes to deliver DNA has been extensively used with a variety of cell types [18]. Encapsulation within a lipid vesicle protects the DNA from hydrolysis and allows transport across the plasma membrane. Large unilamellar vesicles ranging from 0.2 to 4.0 μm can encapsulate up to 40% of an aqueous buffer containing large macromolecules and subsequently deliver them to cells in a biologically active form [19]. The composition of liposome membranes can also be designed to target subpopulations of cells depending on surface receptors [20]. Lipofection is an efficient system of DNA delivery to primary keratinocytes [16].

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Table I. Gene Transfer Technologies

Chemical	DNA-mediated transfection
	Lipofection
Physical	Microinjection
	Electroporation
	Micro-projectiles
Biologic	Retrovirus
	Adeno associated virus
	Adenovirus
	Human papillomavirus
	Epstein-Barr virus

Physical Techniques

Microinjection: Microinjection depends on the physical introduction of genetic material to the nucleus of each cell by use of a fine glass needle. It is the only technique that introduces the DNA directly into the cell nucleus. Although this is a laborious process because each cell has to be individually manipulated, it does permit one to examine expression with the certainty that known numbers of copies of the introduced gene are present in the target cell nucleus. For example, microinjection of mucosal and epidermal keratinocytes with gene constructs containing a promoter from human papillomavirus showed that the promoter was indeed tissue specific [21].

Electroporation: In recent years electroporation has become a method of choice for many cell types that cannot be passaged or that are grown in suspension [22]. This method for gene transfer uses a short high-voltage electric field to induce reversible micropores in the plasma membrane thereby allowing uptake of DNA from the medium [23]. Electroporation has been used to transfect murine keratinocytes [24], human epidermal keratinocytes [25,26], and human cervical keratinocytes [27]. Defining optimal conditions for this technique is not trivial as consistent results have not been found by all investigators [16].

Biologic Expression Vectors The limitations in gene transfer using plasmid DNA are largely overcome with the use of eukaryotic viral vectors. In the last decade remarkable progress has been made in terms of developing retroviral vectors that are useful for efficiently introducing genes into mammalian cells. Likewise, vectors that have been constructed based on four DNA viruses appear promising for use with cultured mammalian cells: adeno-associated virus (AAV), adenovirus (Ad), human papillomavirus, (HPV), and Epstein-Barr virus (EBV).

Eukaryotic plasmid vectors are constructed based on elements from varied cellular and viral sources. They can be categorized as episomal (freely replicating) or integrating (nonreplicating). They also vary in terms of host range and size of insert that can be accommodated **(Table II)**.

Retrovirus: Replication-defective retroviral vectors have been engineered to produce a virus that cannot replicate, but can infect, if encapsidated by viral proteins in specifically designed packaging cells. This is achieved by the replacement of most or all of the coding region(s) of a retrovirus with the gene(s) to be transferred. Detailed protocols for generating infectious retrovectors beginning with the recombinant DNA have been published elsewhere [28].

The amount of DNA that can be inserted in retroviral vectors varies but, generally, up to 10 kb of DNA can be accommodated [29]. Upon infection, viral RNA is reverse transcribed to DNA and, in dividing cells, is integrated as a single copy of proviral DNA [30].

Several variables must be considered in using retroviral vectors to transduce keratinocytes. A key factor in achieving efficient transfer is viral titer. For primary keratinocytes in culture, viral titers greater than 5×10^6 colony forming unit (CFU)/ml result in transduction of all clonogenic cells whereas titers in the range of 10^5 transduce approximately 5% of the clonogenic cells [31]. Transduced keratinocytes harboring a single copy of retrovector DNA can undergo up to 30 cell divisions before senescence. The number of cell divisions suggests that stem cells have been transduced. The significance of this will be discussed later.

Improvements in vector design and in packaging cell lines [32] have led to vectors that yield high titers (10^7/colony-forming units/ml). However, it is also important to keep in mind that insertion of the sequences of interest into the vector may affect viral titers. For example, the presence of a strong polyadenylation signal in the gene being inserted is likely to cause premature termination of the RNA in the packaging cell line and thus reduce viral titers of the full-length viral clones [33].

Primary cells have been successfully infected with retroviral vectors provided the cells are replicating [34]. Until recently, it was assumed that retroviral integration depended on DNA synthesis. Current studies, however, suggest that integration depends on mitosis rather than replication [35]. Whatever the mechanism, optimal transduction rates will be obtained if a sizeable fraction of the population is dividing.

Retroviral vectors have been successfully used for the transduction of keratinocytes, which when grown into a multilayered epithelial sheet can be assayed for secretion of the transduced gene and subsequently used for grafting. Morgan *et al* [36] first showed that primary keratinocytes secreted human growth hormone when transduced with a retrovirus harboring that gene. If transfected into an immortal keratinocyte line this protein could be seen in the bloodstream of grafted mice [37].

Gerrard *et al* [38] have also reported successful transduction of primary keratinocytes. The construct utilized included the human factor IX gene under the control of the murine leukemia virus long terminal repeat with the neo^r gene under the control of an SV40 promoter. The transduced keratinocytes were capable of synthesizing and secreting biologically active factor IX not only in culture but also into the bloodstream of grafted mice. Retroviral vectors are currently being used to transfer and express genes for a variety of uses including for the genetic modification of human somatic cells in order to correct genetic disease [39].

Adeno Associated Virus (AAV): AAV is an animal DNA virus that belongs to the parvovirus family [40]. When AAV is coinfected with either adenovirus or herpes simplex virus it undergoes a productive infection. In the absence of helper virus, AAV establishes a latent infection in which the viral genome integrates into the host genome [41]. Integration is not well understood and seems to occur only at one site in human cells (chromosome 19 q13-qter) [42]. Latent infection can proceed to a normal productive infection if the cell is subsequently infected with helper virus.

The use of AAV as a viral transduction vector has been established for almost ten years [43]. The host range for AAV seems exceptionally broad because every mammalian cell line thus far evaluated can be productively infected with AAV [44]. There is no pathology

Table II. Comparison of Virus-Derived Vectors

	RV	AAV	Ad	HPV	EBV
Integration	Yes	Yes	No	No	No
Host range	Dividing cells	All cells tested	Non-dividing cells	Epithelial cells	Many human cells
Maximum kb load	10	5.2	7.5	?	35
Safety data	Yes	Some	Yes	No	No

associated with AAV and about 85% of normal adults are positive for serum antibodies to AAV capsid proteins [44].

Transduction frequencies of AAV vectors are between 0.5% and 5%. There are limits to how much DNA can be packaged by AAV. Large inserts lower the infection rate, and although up to 5.2 kb can be accommodated, packaging this size is very inefficient. The promiscuity of this vector, coupled to its relatively high transduction rate in human cells make AAV a potentially exciting candidate vector for introducing genes into cultured keratinocytes.

Adenovirus (Ad): Ad vectors have a natural tropism for respiratory and oral epithelial cells [45] although they exhibit a broad host range specificity. During its lytic cycle the virus is taken up by cells, migrates to the nucleus, and does not integrate into the host chromosome. In contrast, cells transformed with Ad have viral sequences stably integrated into the cellular genome and no detectable free virus present.

Replication-defective adenoviruses have been developed as vectors for therapeutic genes. Ad vectors are currently being used and have been engineered to introduce the human gene for the cystic fibrosis transmembrane conductance regulator [46] as well as for alpha-1-antitrypsin into human airway epithelium [47]. In contrast to retroviruses, adenoviruses infect non-dividing cells, a great advantage in targeting quiescent keratinocyte stem cells. By the same token, however, these vectors are likely to infect non-replicating, terminally differentiated cells, limiting expression to the transit times of these cells. In fact transient expression has been one of the main drawbacks to the Ad vector system.

An important safety consideration that cannot be ignored in reference to vectors based on adenoviral sequences is that existing replication-defective adenoviral vectors may be partially replication-competent. The responses that this low level replication may elicit has not been determined [48,49].

Human Papillomaviruses (HPV): HPV are epitheliotropic and are distinguished by the fact that, in benign papillomatous lesions, multiple copies of viral DNA replicate as stable episomes. It is thought that basal cells maintain a low level stable replication of HPV, and a high copy number in more differentiated superficial cells [50]. Genes transferred with a bovine papilloma virus-based vector, which is the most commonly used model for the study of papillomatous DNA replication, generally remain episomal, thus avoiding insertional mutagenesis and providing a multicopy pool of vector genomes for expression [51].

HPV type 1 derived from plantar warts has been used to infect human keratinocytes [52] as has HPV type 11, a genital-derived HPV [53]. HPV type 11 DNA has also been used to transfect cultured human keratinocytes using calcium phosphate precipitation [14]. The fact that long-term episomal replication of HPV DNA was achieved by Mungal *et al* indicates that although they have yet to be developed, HPV-based vectors may prove to be extremely useful for targeting human keratinocytes.

Epstein-Barr Virus (EBV): EBV, a member of the herpesvirus family, normally infects B cells and certain nasopharyngeal cells. EB virions contain a linear DNA molecule of 172 kb that presumably circularizes via its terminal repeats and is thus maintained as an episome in the cell's nucleus [54]. DNA-mediated transfection of portions of this virus have been successful in establishing stably maintained plasmids in many cell lines [55].

EBV-based circular constructs have been used by Jensen *et al* to introduce the sulfatase gene into keratinocytes cultured from patients with X-linked ichthyosis. However, it has not yet been determined whether there is long-term expression resulting from this vector [15]. Recently, an EBV based vector was shown to have high transfection efficiency and to be maintained stably for at least six months in a human embryonic kidney cell line [56]. A potential unique advantage of vectors derived from EBV is their large capacity. Inserts of up to 35 kb in length have been introduced with this vector [55].

Dominant Selectable Genes As has been mentioned, there are a variety of dominant selectable genes that have been transferred either as cotransfectant DNA or as an element within the viral vectors (for recent review [57]). These include resistance to various agents such as G418, hygromycin, methotrexate, puromycin, mycophenolic acid, xanthine, aminopterine, and histidinol (**Table III**). The aminoglycoside phosphotransferase gene (usually referred to as neo^r) confers resistance to G418, a neomycin analogue, and has been useful in keratinocytes.

Selection of keratinocytes is not a simple matter and requires that various provisions be kept in mind. First, selection should begin after the transduced cells have completed at least two cycles of replication to allow time for integration and expression of the newly introduced gene. In our experience it is also advisable to begin G418-selection before individual colonies contain more than 5–10 cells. If the colonies become large, G418-resistant cells linked by desmosomal junctions to neighboring G418-sensitive cells may become dislodged when the sensitive cells become necrotic and are shed into the medium. By keeping colony size to a minimum, loss of neo^r cells by this mechanism seems to be reduced.

Reporter Genes There are a number of reporter genes whose products can be detected histochemically or biochemically and have been very useful in monitoring expression against a low or zero background. One such reporter gene used in keratinocyte studies is the *Escherichia coli* lacZ gene that encodes for β-galactosidase (β-gal). Commercially available antibodies to β-gal make it practical to immunolocalize the enzyme [58]. β-gal activity can also be quantified biochemically by incubating the live cells or a cell lysate in o-nitrophenyl-β-D-galactopyranoside [59]. The reaction product is soluble and can be quantified spectrophotometrically at 420 nm. The reagents in this assay are not toxic over short periods of time and cultivation can be resumed after the assay has been performed. We have found this capability to be very useful in screening large numbers of cultures of transduced primary keratinocytes. Other reporter genes that have been introduced into keratinocytes include CAT, luciferase, neuropeptide P, and SV40 T antigen.

It should be noted that none of the selectable or reporter genes thus far tested seem to have deleterious effects on keratinocyte growth potential or differentiation [60,31]. Furthermore, transgenic mice expressing β-gal in keratinocytes do not appear to undergo changes or transformations when compared to normal mouse keratinocytes [61].

Transduction of Epithelial Stem Cells The replicating population of cells in epidermis is heterogenous. Included in this population are stem cells that are the true progenitor cells and transit amplifying cells that replicate a set number of cycles before differentiating [62].

In culture, the replicating population of cells is also heterogenous [63,64]. It is likely to include stem cells in light of the fact that cultured keratinocytes can form a permanent autologous graft [3] and that clones of transduced newborn foreskin keratinocytes can be expanded routinely to 10^9 and to 10^{10} cells before showing signs of senescence [31]. However, the difficulty of getting stable transfection in keratinocytes may be primarily due to unsuccessful targeting of the stem cell population. Future directions in this regard will be to gauge the long-term phenotype of transduced cells and to enrich for and target stem cells of epidermal tissue [64].

Promoters

Exogenous: An important aspect of both transient and stable gene expression is the choice of promoter to regulate transcription. High levels of transcription have been achieved through the use of a variety of viral promoters that yield constitutively, high levels of gene expression in keratinocytes [36]. However, viral promoters do not usually sustain high levels of expression in transduced keratinocytes. Examples of down-regulation of viral promoters have been observed in keratinocyte lines [37] as well as in normal keratinocytes [38]. Transcriptional silencing of an endogenous promoter located

Table III. Examples of Dominant Selectable Markers [77]

Enzyme	Drug for Selection	Mechanism
Aminoglycoside phosphotransferase (APH)	Geneticin (G418)	APH inactivates G418 (10)
Dihydrofolate reductase (DHFR)	Methotrexate (Mtx)	DHFR resistant to Mtx [78]
Hygromycin-B-phosphotransferase (HPH)	Hygromycin-B	HPH inactivates hygromycin-B [11]
Xanthine-guanine phosphoribosyltransferase (XGPRT)	Mycophenolic acid	XGPRT synthesizes GMP from xanthine [79]
Adenosine deaminase (ADA)	9-β-D-xylofuronosyl adenine	ADA inactivates Xyl-A

downstream of a viral promoter in an expression cassette has also been seen [65].

When genetically transformed cells are transplanted back to the host, expression of genes regulated by exogenous promoters is short lived. A detailed analysis of this phenomenon was carried out by *Palmer et al* [66] with rat fibroblasts transduced with the genes for neo^r and human adenosine deaminase (hADA). The cells were transplanted into isogeneic rats as dermal equivalents. hADA activity was present in the graft at 2 weeks post-transplantation, but by 1 month was undetectable. Semiquantitative PCR analysis of DNA extracted 8.5 months post-transplantation showed that hADA DNA had indeed persisted at undiminished levels. Furthermore, vector positive fibroblasts cultured from the tissue at 8.5 months failed to express the neo^r phenotype. These studies, and others [38,60], indicate that even if transduced cells survive, virally encoded genes are gradually inactivated.

Certain foreign promoters may permit modulated expression in a keratinocyte graft. For example, inclusion of a steroid hormone receptor element in the inserted construct may permit deliberate up-regulation of the encoded gene by topical application of a suitable steroid [67].

Endogenous: The issue of inadequate long-term expression using viral promoters may be solved by the use of endogenous cellular promoters. Although it is possible to utilize an endogenous promoter such as the β-actin, it may be desirable to use tissue-specific promoters to restrict expression to keratinocytes and to avoid promiscuous expression in non-target tissue. The use of a particular endogenous promoter to drive foreign gene expression appears to have no deleterious effect on expression of the endogenous gene from which the promoter was derived [68].

Candidate tissue-specific promoters of genes expressed primarily, or exclusively, in keratinocytes include promoter regions for keratin genes, involucrin, filaggrin, and loricrin. Keratin promoters have successfully been used to direct tissue- and strata-specific expression of foreign genes in transgenic mice [68,69]. In a recent report, transgenic mouse lines were generated using the involucrin promoter linked to the β-gal reporter gene. In the resulting lines, β-gal was expressed in the suprabasal compartment of epithelial cells indicating that the promoter is expressed not only in a strata-specific fashion but also in a tissue-specific expression [70].

It has been suggested that directing gene expression to basal cells may enhance systemic uptake of a secreted gene product [71]. Expression in the basal layers can be achieved through the use of a basal cell-specific promoter such as the keratin 14 promoter. Alternatively, the use of promoters such as that of keratin 1, keratin 10, or involucrin would target suprabasal, more transcriptionally active keratinocyte cell layers that might assure high levels of expression exclusively in stratified epithelia. Finally, it may be useful to use promoters such as that of the keratin 6 that are active during hyperproliferation.

IN VIVO KERATINOCYTE GENE TRANSFER

Techniques to introduce genes directly into animals *(in vivo)* without having to resort to manipulations in culture have been explored. *In vivo* gene transfer in cells other than keratinocytes has been achieved using calcium phosphate precipitation [72], DNA encapsulated within liposomes [73], or by direct injection of DNA and RNA [74]. DNA has also been introduced in mice intrahepatically and intradermally using microprojectiles to shoot the DNA directly into the animal [75].

To date, the only data of *in vivo* gene transfer to keratinocytes was reported by Dennis Roop.* Using an adenovirus vector containing the bacterial β-gal gene at a titer of 5×10^{10} colony-forming units/ml murine keratinocytes were infected *in situ.* In this as well as all the other *in vivo* experiments, high initial levels of expression are observed but expression is short lived and usually disappears within 1 month. Because these types of protocols are used directly on the animal, drug selection and characterization of individual clones are not possible.

Future Directions Transfer and expression of foreign DNA has been accomplished in keratinocytes using a variety of techniques. Nevertheless, none of these techniques have given long term, stable expression of genes introduced into primary keratinocytes. Future developments, particularly with respect to expression vectors, selection, stem cell identification, and stable targeted expression via endogenous promoters must all be addressed. Information on the consequences of over-expression of a foreign gene in keratinocytes is an area of great importance that also must continue to be explored.

The introduction of genes into keratinocytes has conceivable applications that are beyond the scope of this review. The potential of creating genetically modified keratinocytes for therapy is one possibility discussed elsewhere [76]. Introducing foreign DNA into keratinocytes both *in vivo* and *in vitro* holds great promise not only for clinical applications but also for basic research. For example, the question of secretory capacity by keratinocytes and epidermal grafts can be investigated by introducing genes into these cells and assaying for product.

This is an exciting field that undoubtedly will continue to be an area of much research.

Note Added in Proof: Two germane articles have recently appeared on the subject of gene transfer in keratinocytes: 1) Setoguchi Y, *et al:* Recombinant adenoviral vectors have been used to transfer genes into primary cultured keratinocytes and injected directly into skin. *J Invest Dermatol* 102:415–421, 1994; and 2) Staedel C, *et al:* A simple and highly efficient transfection technique for primary cultured keratinocytes using a lipopolyamine: DNA complex has been developed. *J Invest Dermatol* 102:768–772, 1994.

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